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(54) **ANTIMICROBIAL BACTERIA STRAINS AND USES THEREOF**

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(57) **ABSTRACT**

The present invention provides novel antimicrobial bacteria strains and uses thereof to prepare novel antimicrobial agents. Also provided are the novel antimicrobial agents.

2 Claims, 4 Drawing Sheets

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(58) **Field of Classification Search**

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Figure 1

tggagagttt gatcctggct caggacgaac gctggcggcg tgcctaatac atgcaagtcg 60
agcggagaat raargaagct tgcttctttc attcttagcg gcggacgggt gagtaacacg 120
taggcaacct gccctcaagc ttgggacaac taccggaaac ggtagctaata accgaatata 180
tgatttggtc gcctgaacga atytggaaag acggagcaat ctgtcacttg aggatgggcc 240
tgcggcgcgt tagctagtgt gtgaggtaac ggctcaccaa ggcgacgatg cgtagccgac 300
ctgagagggt gatcggccac actgggactg agacacggcc cagactccta cgggaggcag 360
cagtagggaa tcttccgcaa tggacgcaag tctgacggag caacgccgcg tgagtgtatga 420
aggttttcgg atcgtaaagc tctgttgcca gggaagaacg tcygrgttag taactgaact 480
crgagtgacg gtacctgaga agaaagcccc ggctaactac gtgccagcag ccgcggtaat 540
acgtaggggg caagcgttgt ccggaattat tgggcgtaaa gcgcgcgcag gcggcttttt 600
aagtcggtg tcacagccca aggtcaacc ttgggtcgca ctggaaactg gagagcttga 660
gtacagaaga ggaaagtga attccacgtg tagcggtgaa atgcgtagag atgtggagga 720
acaccagtgg cgaaggcgac tttctgggct gtaactgacg ctgaggcgcg aaagcgtggg 780
gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgaatg ctaggtgtta 840
ggggtttcga tacccttggg gccgaagta acacattaag cattccgcct ggggagtacg 900
gtcgcaagac tgaaactcaa aggaattgac ggggacccgc acaagcagtg gagtatgtgg 960
tttaattcga agcaacgcga agaacttac caagtcttga catccctttg accggactag 1020
agatagtcct ttccttcggg acaaaggaga caggtggtgc atggtgtcg tcagctcgtg 1080
tcgtgagatg ttgggttaag tccgcaacg agcgcaaccc ttatgcttag ttgccagcac 1140
atcatggtgg gcactctaag cagactgccg gtgacaaacc ggaggaaggt ggggatgacg 1200
tcaaatcatc atgccccta tgacttgggc tacacacgta ctacaatggc cggatacaacg 1260
ggaagcaata tcgcaagatg gagccaatcc ttaaagccg gtctcagttc ggattgcagg 1320
ctgcaactcg cctgcatgaa gtcggaattg ctagtaatcg cggatcagca tgccgcggtg 1380
aatacgttcc cgggtcttgt acacaccgcc cgtcacacca cgagagtttg caacaccga 1440
agtcggtggg gtaaccgcaa ggagccagcc gccgaaggtg gggtagatga ttggggtgaa 1500
gtcgtaaaca ggtagccgta tcggaaggtg cggytgatc acctcctt 1548

Figure 2

tggagagttt gatcctggct caggacgaac gctggcggcg tgcctaatac atgcaagtcg 60
aacgcttttt ctttcaccgg agcttgctcc aycgaaagaa aaagagtggc gaacgggtga 120
gtaacacgtg ggtaacctgc ccatcagaag gggataacac ttggaacag gtgctaatac 180
cgtataacac tattttccgc atggaagaaa gttgaaaggc gcttttgcgt cactgatgga 240
tggaccccg cggtcattagc tagttgggtga ggtaacggct caccaaggca acgatgcata 300
gccgacctga gaggggtgatc ggccacactg ggactgagac acggcccaga ctctacggg 360
aggcagcagt agggaatctt cggcaatgga cgaaagtctg accgagcaac gccgcgtgag 420
tgaagaaggt tttcggatcg taaaactctg ttgttagaga agaacaagga tgagagtaaa 480
acgttcatcc cttgacggta tctaaccaga aagccacggc taactacgtg ccagcagccg 540
cggtaatacg taggtggcaa gcgttgctcg gatttattgg gcgtaaagcg agcgcaggcg 600
gtttcttaag tctgatgtga aagcccccg ctcaaccggg gagggtcatt ggaaactggg 660
agacttgagt gcagaagagg agagtggaat tccatgtgta gcggtgaaat gcgtagatat 720
atggaggaac accagtggcg aaggcggctc tctggtctgt aactgacgct gaggctcgaa 780
agcgtgggga gcgaacagga ttagataccc tggtagtcca cgccgtaaac gatgagtgtc 840
aagtgttga gggtttccgc cttcagtcgc tgcagcaaac gcattaagca ctccgcttgg 900
ggagtacgac cgcaagggtg aaactcaaag gaattgacgg gggcccgcac aagcgggtga 960
gcatgtggtt taattcgaag caacgcgaag aacctacca ggtcttgaca tccttgacc 1020
actctagaga tagagcttcc cttcggggg caaagtgaca ggtggtgcat ggtgtcgtc 1080
agctcgtgtc gtgagatgtt gggttaagtc ccgcaacgag cgcaaccctt attgttagtt 1140
gccatcattt agttgggcac tctagcgaga ctgccggtga caaaccggag gaaggtggg 1200
atgacgtcaa atcatcatgc cccttatgac ctgggctaca cacgtgctac aatgggaagt 1260
acaacgagtt gcgaagtcgc gaggctaagc taatctctta aagcttctct cagttcggat 1320
tgtaggctgc aactcgccta catgaagccg gaatcgctag taatcgcgga tcagcacgcc 1380
gcggtgaata cgttccccgg cttgtacac accgcccgtc acaccacgag agtttgtaac 1440
acccgaagtc ggtgaggtaa ctttttggg gccagccgcc taaggtggga tagatgattg 1500
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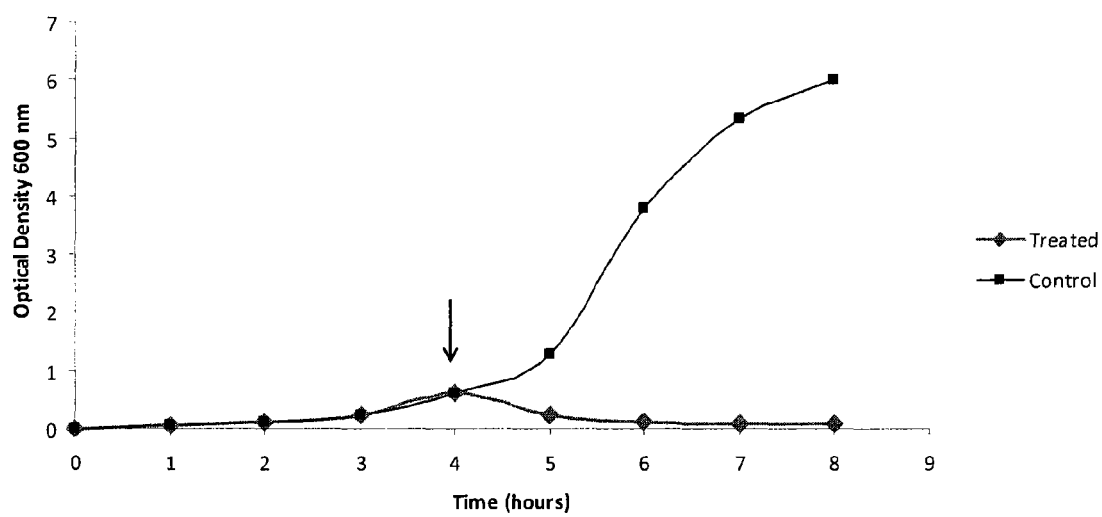
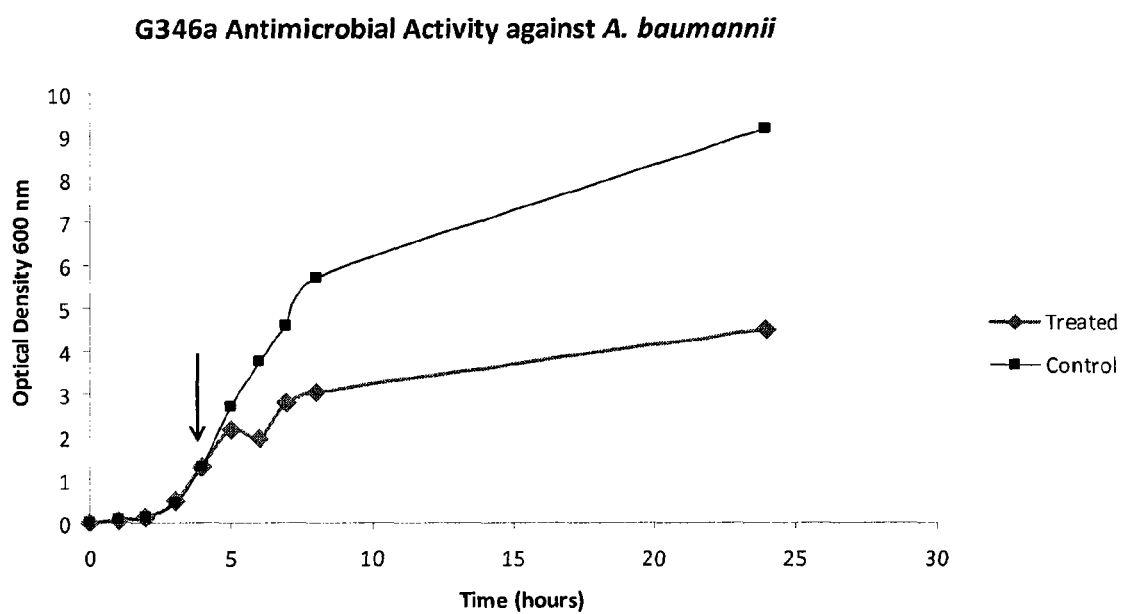
Figure 3**G346a Antimicrobial Activity against *S. aureus***

Figure 4



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ANTIMICROBIAL BACTERIA STRAINS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This patent application is a U.S. National Phase Application of International Application PCT/US2011/033892, filed Apr. 26, 2011, which claims priority to U.S. Provisional Application No. 61/327,939, filed Apr. 26, 2010, the entire contents of which are incorporated by reference in their entirety for all purposes.

FIELD OF THE INVENTION

This invention relates to identification of novel antimicrobial bacteria strains and use of these antimicrobial bacteria strains to prepare novel antimicrobial agents.

BACKGROUND OF THE INVENTION

Over the last few years, antibiotic-resistant bacteria have been isolated in increasing numbers, but anti-microbial drugs are losing their effectiveness. For some infections, such as those caused by methicillin resistant *S. aureus* (MRSA) and multi-drug resistant (MDR) *A. baumannii*, there are virtually no antibiotics available for treatment. For many other infections, the choice of antibiotics is rapidly diminishing.

Based on PCR technology and comparative microbial genome sequencing, it has been estimated that only 1-5% of existing bacteria have been cultured in the laboratory. The ability to cultivate new species in the laboratory offers the ability to isolate novel compounds useful in many industries.

Novel bacteria exhibiting antimicrobial properties are useful in light of the increasing number of infections due to antimicrobial resistance. Because many microbes are resilient and develop resistance, there is a pressing need for the development of new anti-microbial agents by identifying and isolating novel bacteria with antimicrobial properties.

SUMMARY OF THE INVENTION

The present invention relates to novel antimicrobial microorganisms and their uses to prepare novel antimicrobial agents.

An isolated microorganism of the genus *Paenibacillus* is provided as deposited with the American Type Culture Collection (ATCC®) on Apr. 26, 2011, having an ATCC® Patent Deposit Designation of PTA-11858. A functionally equivalent microorganism of the isolated microorganism is also provided. The functionally equivalent microorganism may comprise a 16S rRNA sequence at least 95% identical to SEQ ID NO:1. A biological culture of the microorganism of the present invention, or a functionally equivalent microorganism thereof, is further provided.

In some embodiments, an antimicrobial agent is prepared by using the microorganism of the present invention, or a functionally equivalent microorganism thereof. The antimicrobial agent may be prepared from a biological culture of the microorganism, or a functionally equivalent microorganism thereof. The antimicrobial agent may also be prepared from a supernatant of a biological culture of the microorganism, or a functionally equivalent microorganism thereof. The antimicrobial agent may comprise an amino acid sequence selected from the group consisting of SEQ ID NOS: 3-18. Also provided is a method for preparing an antimicrobial agent. The

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method comprises using the microorganism of the present invention, or a functionally equivalent microorganism thereof.

In some other embodiments, a method for treating a microbial infection in a subject in need thereof is provided. The method comprises administering to the subject an effective amount of an antimicrobial agent. The antimicrobial agent is prepared by using the microorganism of the present invention, or a functionally equivalent microorganism thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the 16S rRNA sequence (SEQ ID NO: 1) of Isolate G346a (ATCC accession number PTA-11858).

FIG. 2 illustrates the 16S rRNA sequence (SEQ ID NO: 2) of Isolate 159.

FIG. 3 illustrates the antimicrobial activity of Isolate G346a (ATCC accession number PTA-11858) against *Staphylococcus aureus* (*S. aureus*).

FIG. 4 illustrates the antimicrobial activity of Isolate G346a (ATCC accession number PTA-11858) against *Acinetobacter baumannii* (*A. baumannii*).

DETAILED DESCRIPTION OF THE INVENTION

While 70 out of 90 antibiotics marketed between 1982-2002 originated from natural sources, the majority (98%) of microorganisms believed to exist have never been cultured. Thus, these previously uncultured microbes offer tremendous potential for new antibiotics. The present invention is based on the discovery of new, previously uncultured microorganisms with antimicrobial activity and the use of these microorganisms to prepare new antimicrobial agents or antibiotics.

According to one aspect of the present invention, microorganisms having an antimicrobial activity are provided. Examples of these antimicrobial microorganisms include bacterial strains identified as novel Isolates Nos. 6, 17, 138, 159, 228, 375, 423, 457, G266, G280, G346a, G505, G507, G522, and G524.

The term "antimicrobial activity" used herein refers to an activity to prevent or inhibit (or reduce) the growth of, or to kill certain microorganisms, including Gram-positive and/or Gram negative bacteria strains, especially those related to currently known antibiotic resistant strains.

Bacterial strains against which these novel isolates have been tested include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Streptococcus pyogenes*. The new strains of bacteria have been identified to exhibit antimicrobial activities against both Gram positive and Gram negative bacteria, and against 3 or more problematic bacterial strains.

Isolate G346a is a microorganism classified as a member of the genus by 16S rRNA sequencing. Its 16S rRNA sequence (SEQ ID NO: 1) as shown in FIG. 1 is 5.30% different from a closest match, *Paenibacillus illinoisensis*. It possesses an antimicrobial activity against a range of Gram positive and Gram negative bacteria.

Isolate 159 is a microorganism classified as a member of the genus

Enterococcus based on 16S rRNA sequencing. Its 16S rRNA sequence (SEQ ID NO:2) as shown in FIG. 2 is 0.18% different from a closest match, *Enterococcus casseliflavus*. It possesses an antimicrobial activity against a range of Gram positive and Gram negative bacteria.

Functionally equivalent microorganisms of the antimicrobial microorganisms of the present invention are also provided. The term "a functionally equivalent microorganism" used herein refers to a microorganism that exhibits essentially the same characteristics and functions as an original microorganism. A functionally equivalent microorganism may be a mutant or variant derived from an original microorganism. The mutant or variant may be formed by various ways (e.g., by UV irradiation or by mutagenic chemicals). The term "essentially the same" used herein refers to being at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical.

A functionally equivalent microorganism of an antimicrobial microorganism is a microorganism that exhibits essentially the same antimicrobial activity, for example, having essentially the same inhibition effect on essentially the same microorganisms, as an original antimicrobial microorganism. A functionally equivalent microorganism may be a mutant or variant derived from an original antimicrobial microorganism. A functionally equivalent mutant or variant may comprise a nucleic acid sequence that is original antimicrobial microorganism (e.g., SEQ ID NO: 1 of Isolate 346a, or SEQ ID NO: 2 of Isolate 159).

A biological, or liquid, culture of an antimicrobial microorganism of the present invention, or a functionally equivalent microorganism thereof, may be prepared by growing the microorganism, or the functionally equivalent microorganism, in a suitable culture medium. The techniques and suitable culture media for preparing a biological culture of a microorganism (e.g., a bacteria strain) are well known in the art.

According to another aspect of the present invention, an antimicrobial agent is provided. The term "an antimicrobial agent" used herein refers to an agent that exhibits an antimicrobial activity. The agent may be a chemical compound, a biological molecule (e.g., a nucleic acid, peptide or protein), or a combination thereof. The antimicrobial agent may be prepared by using an antimicrobial microorganism of the present invention, or a functionally equivalent microorganism thereof. For example, an antimicrobial agent may be prepared by purification and/or isolation from a biological culture, or a supernatant of the biological culture, of the microorganism, or a functionally equivalent microorganism thereof. The antimicrobial agent may comprise an amino acid sequence selected from the group consisting of SEQ ID NOs: 3-18. Also provided is a method for preparing an antimicrobial agent comprising using the antimicrobial microorganism of the present invention, or a functionally equivalent microorganism thereof.

According to a further aspect of the present invention, a method for treating a microbial infection in a subject in need thereof is provided. The method comprises administering to the subject an effective amount of the antimicrobial agent of the present invention. The subject is a mammal, for example, a mouse, rat, dog, pig or human, preferably a human. The effective amount will depend on the route of administration,

the nature of the formulation, and the subject's condition. The antimicrobial agent may be formulated, for example, for an oral, sublingual, intranasal, intraocular, rectal, transdermal, mucosal, topical or parenteral administration.

The term "about" as used herein when referring to a measurable value such as an amount, a percentage, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

EXAMPLE 1

Antimicrobial Libraries

Microbial libraries of microorganisms containing previously unculturable species were established using quorum quenching technology, which disrupts the quorum sensing ability of bacteria. Each library consists of bacteria from several different natural sources, including: freshwater (pond), soil (corn field, grassy field), sewage, rumen fluid (cow), and compost pile (aerobic and anaerobic).

The libraries were screened for an antimicrobial activity. Microbial isolates of the libraries were screened for an antimicrobial activity using a Biological Growth Inhibition assay against the following indicator strains: *Acinetobacter baumannii*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. Library isolates were spotted on a lawn of the indicator organism. Plates were incubated for 24-48 hours and activity was observed as a zone of inhibition around the bacterial spot.

E. coli and *Bacillus* strains which contain antibiotic resistant plasmids were also screened against to discriminate lead antimicrobial agents, including compounds, from the following previously described antibiotics: ampicillin, kanamycin, streptomycin, spectinomycin, tetracycline, chloramphenicol, and erythromycin.

Novel bacteria were isolated in combination with the quorum-quenching enzyme Esterase, from porcine liver (Sigma E3019-20KU, 17 units/mg solid) as described in

Example 1 in U.S. Pat. No. 7,494,810. According to 16S rRNA sequencing, approximately 50% of all characterized isolates were new species. As a result, novel bacteria were identified, after isolation, to contain an antimicrobial activity.

EXAMPLE 2

Antimicrobial Microorganisms

Approximately 100 isolates produced an antimicrobial activity against 3 or more problematic bacterial strains, both Gram positive and Gram negative, and were considered isolates of interest. Fifteen of these isolates, identified in TABLE 1, exhibited characteristics of special interest and were sent for 16S rRNA sequencing (by Accugenix, Newark Del., using conventional techniques) to determine their closest match with 16S rRNA sequences of known bacteria.

TABLE 1

Isolate #	Closest Match, % Difference, Taxonomic Confidence Level	<i>S.a.</i> G+	<i>E. coli</i> G-	<i>Pseudo</i> G-	<i>K.p.</i> G-	<i>A.b.</i> G-	<i>E.f.</i> G+	<i>Salmo</i> G-	<i>List.</i> G+	<i>Strep.</i> G+
6	<i>Serratia-proteamaculans-quinovora</i> 1.23% Genus	0.4	0.2				0.25	0.1		
17	<i>Pseudomonas-alcaligenes</i> 1.15% Genus	0.5	0.4		0.15	0.35	0.2	0.2	0.25	0.2
138	<i>Paenibacillus-amyolyticus</i> 6.03% No Match	0.6				0.1	0.3		0.2	0.8
159	<i>Enterococcus-casseliflavus</i> 0.28% Species	1	0.4	0.3	0.05	0.1		0.1		
228	<i>Burkholderia-plantarii</i> 2.00% Genus		0.3		0.15	0.3		0.4		
375	<i>Pseudomonas-fulva</i> 1.05% Genus	0.4	0.3	0.1	0.15	0.3	0.1	0.2		0.5
423	<i>Paenibacillus-amyolyticus</i> 6.22% No Match	0.6				0.1	0.3		0.3	0.7
457	<i>Pseudomonas-asplenii/putida</i> 1.53% Genus	0.2	0.3		0.1	0.05	0.15	0.15		0.3
G266	<i>Pantoea-agglomerans</i> 2.46% Genus	0.7	1	0.1	0.05	0.5	0.7	0.3		0.1
G280	<i>Pantoea-ananatis</i> 2.28% Genus	1	1	0.1	0.05	0.4	0.6	0.2		0.1
G346a	<i>Paenibacillus-illinoisensis</i> 5.30% Genus	0.6				0.15	0.3		0.25	0.7
G505	<i>Enterobacter-asburiae</i> 1.33% No Match	0.3	0.3	0.05			0.3	0.4	0.25	0.3
G507	<i>Enterobacter-asburiae</i> 1.52% No Match	0.3	0.3	0.05			0.3	0.4	0.25	0.3
G522	<i>Enterobacter-asburiae</i> 1.43% No Match	0.3	0.3	0.05			0.3	0.4	0.25	0.3
G524	<i>Enterobacter-asburiae</i> 1.24% No Match	0.3	0.3	0.05			0.3	0.4	0.25	0.3

TABLE 1 lists 15 novel isolates that demonstrate a broad range of antimicrobial activities against three or more of the problematic bacterial strains (both Gram positive and Gram negative) listed at the top of columns 3-11 in TABLE 1. TABLE 1 also lists, with reference to the novel isolates, their closest related species, and the level of antimicrobial activities of the various isolates. More specifically, for each isolate, TABLE 1 indicates its isolate number, Closest Match, Percentage Difference to the Closest Match, and taxonomic confidence level from sequencing, and antimicrobial activities against specific problematic bacterial strains measured in centimeters. This centimeter measurement is the Zone of Inhibition and measures the distance from the perimeter of the bacteria to the perimeter of the clear zone demonstrating an antimicrobial activity exhibited by each corresponding isolate.

Closest Match is the closest match of a related species to the sample when aligned in pairwise manner against the Sherlock Database.

The Percentage Difference to the Closest Match is the percent difference between the sample and the Closest Match. Mismatched basepairs, gaps, and ambiguity codes are all accounted for in this percentage.

Three Confidence Levels, representing the closeness of the novel isolate to the Closest Match, are identified in TABLE 1 as: (1) "Species," in which the difference between the novel isolate and the Closest Match is $\leq 1.00\%$ (2) "Genus," in which the difference is $\leq 5.00\%$ and (3) "No Match" with a Percentage Difference greater than 5%.

The following are the nine problematic bacterial strains abbreviated in TABLE 1 to which isolates exhibited resistance:

- Staphylococcus aureus* (Abbreviated S.a.)
- Escherichia coli* (Abbreviated E. coli)
- Pseudomonas aeruginosa* (Abbreviated Pseudo)
- Klebsiella pneumonia* (Abbreviated K.p.)
- Acinetobacter baumannii* (Abbreviated A.b.)
- Enterococcus faecalis* (Abbreviated E.f.)
- Salmonella typhimurium* (Abbreviated Salmo)
- Listeria monocytogenes* (Abbreviated List.)
- Streptococcus pyogenes* (Abbreviated Strep.)

Isolates G346a and 159 and their supernatants have been studied in more detail as described below.

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EXAMPLE 3

Isolate G346a

Isolate G346a was analyzed to determine the Zone of Inhibition resulting from its antimicrobial activity, and to determine the activity of its supernatant, temperature stability and pH stability. The Zone of Inhibition was measured as the distance from the perimeter of the bacteria to the perimeter of the clear zone and recorded in centimeters.

a. Bacterial Screen

TABLE 2 illustrates the Zone of Inhibition of the G346a Isolate against the bacterial strains tested in Example 2.

TABLE 2

Bacterial Strain	Zone of Inhibition (cm)
<i>Staphylococcus aureus</i>	0.6
<i>Escherichia coli</i>	0.1
<i>Pseudomonas aeruginosa</i>	neg
<i>Klebsiella pneumonia</i>	neg
<i>Acinetobacter baumannii</i>	0.1
<i>Enterococcus faecalis</i>	0.4
<i>Salmonella typhimarium</i>	neg
<i>Listeria monocytogenes</i>	0.3
<i>Streptococcus pyogenes</i>	0.8

A “neg” result in the Bacterial ZOI (Zone of Inhibition) indicates no clear zone and no antimicrobial activity.

b. Supernatant

The antimicrobial activity of the supernatant of the G346a isolate was then determined. The isolate was grown in a biological, or liquid, culture, spun and filtered and then an antimicrobial screen was performed as follows:

1. Inoculate 20 μ l bacterial suspension of G346a (OD₆₀₀=0.5) into 20 mls Terrific Broth.
2. Incubate at 32° C., shaking at 200 rpm for 24 hours.
3. Centrifuge bacterial culture at 10,000 rpm for 30 mins.
4. Sterilize supernatant using 0.4 μ filter apparatus.

TABLE 3

Bacterial Strain	Bacterial ZOI (cm)	Supernatant Activity
<i>S.a.</i>	0.6	pos
<i>E. coli</i>	0.1	neg
<i>Pa.</i>	neg	neg
<i>K.p.</i>	neg	neg
<i>A.b.</i>	0.1	neg
<i>E.f.</i>	0.4	pos
<i>Salmo</i>	neg	neg
<i>List.</i>	0.3	pos
<i>Strep</i>	0.8	pos

TABLE 3 sets forth the Bacterial Zone of Inhibition and Supernatant Activity of the G346a Supernatant. A “neg” result in the Bacterial ZOI (Zone of Inhibition) indicates no clear zone and no antimicrobial activity.

c. Temperature Stability

The Temperature Stability of the G346a supernatant was tested against a control. The G346a supernatant was incubated at different temperatures for 24-72 hours to observe stability. The G346a supernatant was plated on a S.a. bacterial strain plate and the plate incubated overnight. The results are shown in TABLE 4. A “pos” indicates a positive result where the G346a supernatant exhibited antimicrobial activity at the corresponding temperature and time. A “neg” indicates a negative result where the G346a supernatant exhibited no antimicrobial activity at the corresponding temperature and time.

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TABLE 4

Temperature	24 hrs	48 hrs	72 hrs
4° C.	pos	pos	pos
28° C.	pos	pos	pos
37° C.	pos	neg	neg
50° C.	partial	neg	neg
70° C.	neg	neg	neg
Control	pos	pos	pos

d. pH Stability

The pH stability of the G346a supernatant was tested. The pH of G346a supernatant was adjusted to pH 3 and pH 10 using 1 M HCl and 1M NaOH. The G346a supernatant was incubated at 37° C. for 2 hours and neutralized to the original pH (7.3) before screening. 20 μ l of the G346a supernatant was plated on a S.a. bacterial strain plate to observe the effect of pH on the supernatant. The results indicated that the G346a supernatant retains full activity at pH 3 (acidic conditions) but does not retain full activity at pH 10 (basic conditions).

e. Antimicrobial Activity Against *Staphylococcus Aureus*

Liquid cultures of *Staphylococcus aureus* were grown for eight hours and the optical density of the cultures was taken every hour. After four hours, at the exponential growth phase, G346a supernatant was added to two samples of the *Staphylococcus aureus* culture. The experiment was carried out in duplicate. FIG. 3 illustrates the average bacteriolytic effect when the G346a supernatant was added during the exponential growth phase compared to two control samples to which G346a was not added.

f. Antimicrobial Activity Against *Acinetobacter Baumannii*

Liquid cultures of *Acinetobacter baumannii* were grown for twenty-four hours and the optical density of the cultures was taken every hour for eight hours and again after twenty-four hours of growth. After four hours, at the exponential growth phase, G346a concentrated supernatant was added to two samples of the *Acinetobacter baumannii* culture. The experiment was carried out in duplicate. FIG. 4 illustrates the bacteriostatic effect when the G346a concentrated supernatant was added during the exponential growth phase compared to two control samples to which G346a was not added.

g. Antimicrobial Agent

A concentrated antimicrobial compound (CAC) was prepared as an antimicrobial agent. 2.1 L of an overnight bacterial culture of G346a was centrifuged at 10,000 \times g for 20 min, and then subject to 0.2 μ m filtration. The supernatant was subject to 10-15% TCA precipitation for 1 hour, and then centrifugation at 4,000 \times for 20 min. The pellet was re-suspended in 3 mL 50 mM NH₄HCO₃, and then neutralized with 320 μ L NH₄OH. The resulting concentrated antimicrobial compound (CAC) was tested for its antimicrobial activity against susceptible and resistant strains as shown in TABLE 5.

TABLE 5

Compound	<i>S. aureus</i>	<i>S. aureus</i> (MRSA)	<i>A. baumannii</i>	<i>A. Baumannii</i> (MDR)
G346a sup.	1:20	N/A	No inhibition	N/A
G346a CAC	1:1,280	1:1,280	1:80	1:320
Ampicillin (1.28 mg/ml)	1:5,120	No Inhibition	1:20	No Inhibition
Carbenicillin (1.28 mg/ml)	1:320	No Inhibition	1:20	No Inhibition
Doxycycline (1.28 mg/ml)	1:5,120	1:5,120	1:5,120	1:640

The antimicrobial agent may further be purified and/or isolated from the concentrated antimicrobial compound

(CAC) of G346a using techniques conventionally known in the art. The components of the antimicrobial agent may be determined using suitable analysis (e.g., mass spectrometry analysis). For example, the CAC of G346a was purified using a 5-ml HiTrap™ hydrophobic interaction chromatography (HIC) column prepacked with Phenyl Sepharose™ High Performance (HP). Active fractions were lyophilized, and further purified using a C18 Reverse-Phase (RP) HPLC column. A single peak of interest was observed, and subject to mass spectrometry analysis. The LC/MS results indicate two potential peptide components in the peak with molecular masses of 3348.2 Da and 5961.9 Da. These two components were further analyzed using MS/MS techniques, and found to contain one of the following amino acid sequences:

Sequence	SEQ ID NO
KFIKSI	3
KFIKSL	4
KFIQSI	5
KFIQSL	6
KFLKSI	7
KFLKSL	8
KFLQSI	9
KFLQSL	10
QFIKSI	11
QFIKSL	12
QFIQSI	13
QFIQSL	14
QFLKSI	15
QFLKSL	16
QFLQSI	17
QFLQSL	18

EXAMPLE 4

Isolate 159

Isolate 159 was also tested to determine the Zone of Inhibition resulting from its antimicrobial activity, and to determine its supernatant activity, temperature stability and pH stability.

a. Bacterial Screen

TABLE 6 illustrates the Zone of Inhibition of Isolate 159 against the bacterial strains tested in Example 2.

TABLE 6

Bacterial strain	Zone of Inhibition (cm)
<i>Staphylococcus aureus</i>	1
<i>Escherichia coli</i>	0.4
<i>Pseudomonas aeruginosa</i>	0.3
<i>Klebsiella pneumonia</i>	0.05
<i>Acinetobacter baumannii</i>	0.1
<i>Enterococcus faecalis</i>	neg
<i>Salmonella typhimarium</i>	0.1
<i>Listeria monocytogenes</i>	neg
<i>Streptococcus pyogenes</i>	neg

A “neg” result in the Bacterial ZOI (Zone of Inhibition) indicates no clear zone and no antimicrobial activity.

b. Supernatant

The antimicrobial activity of the supernatant of isolate 159 was then determined. The isolate was obtained via a Cross-Species induction method active against *Pseudomonas aeruginosa* (P.a.) in order to induce production of the antimicrobial, after which the 159/P.a. isolate was grown in liquid culture, spun and filtered and used for an antimicrobial screen. The process was conducted as follows:

1. a. Inoculate 20 μ l bacterial suspension of 159 (OD600=0.5) into 20 mls Terrific Broth.
- b. Separately, inoculate 20 μ l bacterial suspension of *Pseudomonas aeruginosa* (OD600=0.5) into 20 mls Terrific Broth.
2. Incubate both cultures at 32° C., shaking at 200 rpm for 8 hours.
3. Add 200 μ l *Pseudomonas aeruginosa* culture to 159 culture.
4. Incubate mixed culture for total period of 24 hours.
5. Centrifuge bacterial culture at 10,000 rpm for 30 mins.
6. Sterilize supernatant using 0.4 μ filter apparatus.

A positive result was exhibited for supernatant activity in the presence of *Pseudomonas aeruginosa*.

c. Temperature Stability

The Temperature Stability of the 159/P.a. supernatant was tested against a control. The 159/P.a. supernatant was incubated at different temperatures for 24-72 hours to observe stability. The 159/P.a. supernatant was plated on P.a. bacterial strain plate and the plate incubated overnight. The results are shown in TABLE 7. A “pos” indicates a positive result where the 159/P.a. supernatant exhibited an antimicrobial activity at the corresponding temperature and time. A “neg” indicates a negative result where the 159/P.a. supernatant exhibited no antimicrobial activity at the corresponding temperature and time.

TABLE 7

Temperature	24 hrs	48 hrs	72 hrs
4° C.	pos	pos	pos
28° C.	pos	pos	pos
37° C.	pos	neg	neg
50° C.	partial	neg	neg
70° C.	neg	neg	neg
Control	pos	pos	pos

d. pH Stability

The pH stability of the 159/P.a. supernatant was tested. The pH of 159/P.a. supernatant was adjusted to pH 3 and pH 10 using 1 M HCl and 1M NaOH. The 159/P.a. supernatant was incubated at 37° C. for 2 hours and neutralized to the original pH (7-7.8) before screening. 20 μ l of the 159/P.a. supernatant was plated on P.a. bacterial strain plate to observe the effect of pH on the supernatant. The results indicated that the 159/P.a. supernatant is not effected by basic conditions.

Although illustrated and described herein with reference to certain specific embodiments, the present invention is nevertheless not intended to be limited to the details shown. Rather, various modifications may be made in the details within the scope and range of equivalents of the claims and without departing from the spirit of the invention.

SEQUENCE LISTING

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What is claimed:

1. A method for preparing an antimicrobial agent, comprising

(a) growing a microorganism in a culture medium, wherein the microorganism has an ATCC®Patent Deposit Designation of PTA-11858, and

(b) purifying or isolating the antimicrobial agent from the culture medium of step (a).

2. The method of claim **1**, further comprising (c) administering the antimicrobial agent to a subject having a microbial infection in an amount effective for treating the microbial infection.

* * * * *